A COMPARISON OF TWO AND THREE DAYS INCUBATION FOR ENUMERATING RAW-MILK BACTERIA[1]

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Abstract

Eighty-three samples of raw milk were assayed by the Standard Plate Count method with incubation periods of two and three days. The three-day incubation period gave higher counts ($0.05 > p > 0.01$). There were no appreciable problems (such as drying-out, spreaders, molds, etc.) encountered when plates were incubated the extra day. An optional three-day incubation period for the Standard Plate Count is recommended.

Standard Methods, 12th edition, *(3)* specifies an incubation temperature of 32± 1 C for 48± 3 hr when assaying milk or milk products for bacteria by the standard agar plate method. The 11th edition of *Standard Methods for the Examination of Dairy Products (2)*, also specified a 48-hr incubation period for raw milk but recommended a 72-hr incubation for dried milks. The longer incubation period for the dried milk was advocated by a Committee set up by the International Dairy Federation (see review) *(7)*. The change back to two days was made after studies by Pedraja *(9)* indicated that although counts were greatly increased after three days of incubation, the grade classification *(1)* was only seldom influenced. The effect of three days incubation on raw milk bacteria was studied by Babel, et al. *(4)*. These workers found no difference in counts when plates were incubated at 32 C for two or three days but did find higher counts at five days. Pasteurized milk, however, showed counts at three days to be higher than at two days. This was also the finding of Nelson and Baker *(8)*.

It is often necessary or desirable to assay milk samples on a Friday—a two-day incubation time would necessitate making plate counts on a Sunday whereas three days of incubation would permit observations to be made on a regular working day. The study reported here was intended to further investigate the effect of a three-day incubation period on plate counts of raw-milk bacteria with a special emphasis on the possible development of undesirable characteristics in the over-incubated plates which might make enumeration more difficult or less precise. Statistical evaluation of the two incubation periods was made with an analysis of differences of mean counts and variances encountered.

Materials and Methods

The study was conducted by nine subcommittee members, each of whom collected his own raw-milk samples either from farm bulk tanks or from holding tanks at the processing plants. There were two separate analyses, with different milk samples and different analysts from the laboratory of investigator D. These were considered to be of equal weight in the statistical evaluation. Another laboratory, that of investigator G, on the other hand, reported the results of analyses of the same milk samples by two analysts; thus providing an opportunity to determine a possible interaction between analysts and samples. The assay methods were those recommended by *Standard Methods (3)* except that a three-day incubation period was included with the same plates being counted at both two and three days. All plates were poured in duplicate. Statistical evaluation was in general similiar to that of previous studies by the Subcommittee *(5, 6)*.

Results

Mean counts

The means of all samples tested are shown in

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TABLE 1. COMPARISON OF TWO AND THREE DAYS INCUBATION ON PLATE COUNTS

Investigator no.	Milk sample no.	Incubation time 2 days		3 days		Δ[1] %
A	1	4.5	5.2	4.7	5.8	8.2
	2	4.2	6.2	6.5	4.0	1.0
	3	6.5	5.6	6.7	5.8	3.3
	4	9.9	10.0	9.9	10.4	2.0
	5	4.4	6.1	4.8	6.5	7.6
	6	22.7	24.9	23.3	25.8	3.2
	7	6.9	7.7	7.3	7.9	4.1
	8	9.0	9.5	9.3	9.7	2.7
Average		8.96		9.28		3.6
B	9	4.2	3.9	4.5	4.0	4.9
	10	16.0	18.3	17.5	19.5	7.9
	11	11.0	11.8	11.3	12.0	2.2
	12	8.7	8.3	9.4	9.1	8.8
	13	4.8	4.5	4.9	4.8	4.3
	14	15.6	15.1	16.9	16.0	7.2
	15	4.5	4.3	4.7	5.1	11.4
	16	5.1	4.9	5.4	5.3	7.0
Average		8.81		9.40		6.7
C	17	111.0	119.0	106.0	111.0	−5.7
	18	980.0	1000.0	910.0	1100.0	1.5
	19	228.0	286.0	240.0	272.0	−0.4
	20	9.2	12.0	10.2	11.8	3.8
	21	2.9	2.8	3.7	3.6	10.5
	22	4.8	2.8	4.9	3.9	15.8
	23	2.9	4.0	3.2	4.5	11.6
	24	5.8	6.3	6.4	6.8	9.1
Average		173.59		174.88		0.7
D_1	25	13.7	15.1	13.8	15.4	1.4
	26	8.2	9.9	8.4	9.4	−1.6
	27	239.0	290.0	252.0	284.0	1.32
	28	7.4	7.0	8.5	6.6	4.9
	29	14.7	14.6	14.8	15.1	2.0
	30	8.2	7.5	8.7	7.4	2.5
	31	61.0	67.0	57.0	71.0	0
	32	24.7	24.6	24.0	27.3	4.0
Average		50.79		51.46		1.3
D_2	33	6.4	7.3	7.0	7.6	6.6
	34	12.1	11.5	12.2	12.8	5.9
	35	7.2	9.2	7.1	9.7	2.4
	36	31.0	49.0	42.0	47.0	11.2
	37	21.0	19.5	20.0	22.6	6.4
	38	4.0	3.8	5.3	5.2	34.6
	39	14.2	13.7	15.0	14.0	3.9
	40	9.2	8.9	9.8	9.7	7.7
Average		14.25		15.44		8.4
E	41	13.4	11.0	10.7	13.3	−1.6
	42	6.0	5.4	7.4	5.8	15.8
	43	7.1	6.8	7.2	6.9	1.4
	44	85.0	96.0	85.0	109.0	7.2
	45	2.3	2.6	2.3	2.7	2.0
	46	127.0	129.0	124.0	132.0	0
	47	9.9	10.1	11.2	11.3	12.5
	48	140.0	130.0	145.0	144.0	7.0
	49	40.0	36.0	47.0	37.0	10.5
	50	16.7	15.2	14.5	15.9	−4.7
	51	615.0	650.0	647.0	650.0	2.5
	52	27.0	29.0	25.5	27.5	−5.4
Average		92.10		95.09		3.2
F	53	22.7	22.5	23.5	23.4	3.8

	54	8.7	10.0	9.3	11.0	8.6
	55	7.9	9.4	9.4	9.8	11.0
	56	3.8	3.4	4.4	3.5	9.7
	57	1.3	1.6	1.4	1.8	10.3
	58	5.6	5.6	6.5	5.8	9.8
	59	4.1	4.1	4.3	4.2	3.6
	60	3.3	3.4	4.1	3.7	16.4
Average		7.33		7.88		7.5
G_1[2]	61	203.0	210.0	236.0	235.0	14.0
	62	1.3	0.8	1.6	1.3	38.1
	63	9.1	9.6	9.7	10.3	7.0
	64	127.0	146.0	163.0	156.0	16.8
	65	8.1	7.8	9.1	10.2	21.4
	66	44.0	72.0	51.0	71.0	5.2
	67	10.0	10.5	11.7	14.5	27.8
Average		61.37		70.03		14.1
G_2	61	221.0	204.0	246.0	219.0	9.4
	62	2.6	2.0	2.5	2.0	−2.2
	63	10.8	12.0	12.6	12.5	10.1
	64	143.0	217.0	171.0	232.0	11.9
	65	9.1	10.1	10.4	11.0	11.4
	66	66.0	56.0	72.0	63.0	10.6
	67	14.7	12.1	15.1	14.4	10.1
Average		70.31		77.39		10.5
H	68	5.9	3.3	6.2	4.8	19.6
	69	7.3	6.2	6.9	6.2	−3.0
	70	6.7	6.9	7.7	8.0	18.0
	71	10.9	12.3	11.5	12.6	3.9
	72	4.0	4.0	5.6	5.6	40.0
	73	39.0	38.0	43.0	42.0	10.3
	74	10.1	10.4	13.7	13.8	34.1
	75	13.3	13.0	14.5	13.4	6.1
Average		11.96		13.47		12.6
I	76	65.0	35.0	66.0	35.0	1.0
	77	7.6	8.6	7.1	10.3	7.4
	78	125.0	123.0	117.0	127.0	−1.6
	79	130.0	136.0	170.0	132.0	13.5
	80	87.0	86.0	97.0	120.0	25.4
	81	21.0	22.2	22.2	22.0	1.8
	82	23.6	26.2	26.7	23.3	0.4
	83	23.5	22.5	23.3	24.0	2.8
Average		58.89		63.92		8.6
Overall Average		52.24		54.88		5.0

[1]Change (Δ) was positive unless otherwise noted.
[2]Analysts G_1 and G_2 took different aliquots of same milk samples and plated and counted independently.
For actual counts multiply by 1000.

Table 1. There was an increase in plate counts at three days with each investigator with the increases ranging from 0.7% for investigator C to 14.1% for investigator G_1. The overall mean difference was 5.0% in favor of the three-day incubation period. Only nine of the analyses showed lower counts at three days than at two. This included the results of analysts G_1 and G_2 who assayed the same milk samples. The greatest individual sample gain was 40% with investigator H and milk sample number 72. Analysts G_1 and G_2, although assaying the same milk samples (using separate dilutions and plates), showed differing degrees of change from two to three days incubation. In one instance, milk sample number 62, a decrease of 2.2% was noted for one analyst and an increase of 38.1% for the other.

Statistical evaluation of plate counts

A non-parametric sign test was made counting the number of times three days incubation was superior to two days and the number of times two days was superior. The test showed that the increase in three days was significant at the 1% level of probability.

An analysis of variance of the $\log_{10}$ transformed counts is shown in Table 2. The largest source of variation was in the milk samples themselves; the differences were significant with $p < 0.01$. The

TABLE 2. ANALYSIS OF VARIANCE OF PLATE COUNTS

	Source of variation	df	Sum of squares	Mean squares	F ratio[1]	Significant at $p < 0.05$	Significant at $p < 0.01$
a	Investigators	9	24.7931	2.7547	2.27	yes	no
b	Samples within investigator	73	88.4660	1.2118	504	yes	yes
c	Days	1	0.0869	0.0869	6.6	yes	no
d	Days times investigators	9	0.1191	0.0132	13.2	yes	yes
e	Days times samples within investigators	74	0.0779	0.0010	0.42	no	no
f	Error (between duplicate plates)	166	0.4114	0.0024			
	Total	331	113.9544				

[1]F ratios obtaining using lines a/b, b/f, c/d, d/e, e/f

TABLE 4. ANALYSIS OF VARIANCE SUMMARY OF VARIANCES DUE TO INVESTIGATORS AND DAYS OF INCUBATION

Source of variation	df	Sum of squares	Mean squares	F ratio	Significant at $p < 0.05$	Significant at $p < 0.01$
Investigators	9	1.9808	0.2201	4.32	yes	no
Investigator B vs. others	1	1.0775	1.0775	21.13	yes	yes
Days	1	0.0108	0.0108	<1.0	no	no
Days times investigators	9	0.5101	0.0510			
Total	19					

treatments (days) showed a significantly higher count ($p < 0.05$) at three days than at two. Investigators were also significantly different with $p < 0.05$ but not with $p < 0.01$. There was a significant interaction effect ($p < 0.01$) between days of incubation and investigators.

Statistical evaluation of variabilities

Table 3 shows the averages of single-degree-of-freedom variances for days of incubation and investigators. Investigator B in previous experiments showed the lowest variability of any other investigator; this was also true in these studies. A further study of the significance of these variance differences was by an analysis of variance of $\log_{10}$ transformed variances of Table 3. The results are summarized in Table 4. The variances (an indication of reproducibility) were not different for two or three days incubation but the investigators did show significant differences in reproducibility with $p < 0.05$ but not with $p < 0.01$. Since most of this difference was suspected as being the low variance (high reproducibility) of investigator B, an orthogonal contrast was made between this investigator and the other nine. The F ratio of this contrast was significant with $p < 0.01$ indicating that this investigator has a lower variability than the others.

Each investigator was given a form to fill out with space for comments on any difficulties encountered (such as drying-out, spreaders, molds, etc.) by incubating the plates for an extra day. No comments were made by six of the participants indicating, presumably, that they encountered no problems. One investigator reported a slight increase in spreaders in two of the plates and slight dehydration in a third but these did not interfere with the counting procedure. Two other investigators reported some mold growth at three days in one sample but again, counts were reported without any apparent difficulties encountered. Thus, out of the 83 milk samples and 166 plates only five plates showed signs of conditions which might interfere with normal counting procedures. One investigator reported that counts

TABLE 3. VARIANCE ESTIMATES OF PLATE COUNTS[1]

Laboratory number	Incubation period: Two days	Incubation period: Three days	Average variance
A	0.003825	0.004862	0.004344
B	0.000462	0.000475	0.000469
C	0.006238	0.002912	0.004575
D_1	0.001200	0.002288	0.001744
D_2	0.003538	0.001638	0.002588
E	0.000908	0.002133	0.001521
F	0.001250	0.001988	0.001619
G_1	0.006800	0.002914	0.004857
H	0.004500	0.001100	0.002800
I	0.004912	0.007962	0.006437
Average variance	0.003363	0.002827	0.003095

[1]These variance estimates (standard deviation squared) were calculated from the pooled single degree-of-freedom variances between duplicate plates using $\log_{10}$ counts.

were easier the third day, since the colonies had increased in size. Another reported that pinpoint colonies had developed on the third day which were not apparent on the second day of incubation. It appeared that incubation for the extra day did not cause any appreciable difficulty in the standard plate method.

Discussion

The results of this experiment showed that there were great differences between investigators and between milk samples in the variations observed. These same variations were observed before *(5, 6)* and emphasize the need for enlisting the help of several laboratories, each assaying a number of milk samples, when changes in analytical methods are being contemplated. The increase in average counts at three days between the different laboratories participating in this study ranged from a negligible 0.7% for investigator C to a considerable 14.1% for investigator G_1. Erroneous conclusions although not serious in this instance, could easily have been reached if investigator C, for instance, had been the only participant. There was a significant interaction between treatments (days) and investigators in this study but no interactions between days and samples within investigators. Previous reports *(5, 6)* indicated significant interactions between investigators and treatments and between treatments and samples within investigators.

The results of analysts G_1 and G_2 also indicated that different investigators may get different treatment effects from the same milk samples, however, it should be noted that the most striking example of analyst differences was with milk sample number 62 which had the lowest count of the seven tested. The plate counts of this sample were < 30 colonies per plate and large inaccuracies would be expected. If enough random samples are chosen these effects can be weeded out and successful conclusions can be made.

We would recommend on the basis of these studies that an optional three-day incubation period be allowed for the standard agar plate method for enumerating raw-milk bacteria. The 5% difference in counts at three days would ordinarily be well within the limits of experimental error.

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References

1. American Dry Milk Institute. 1963. Bulletin 916-Standards for grades for the dry milk industry, including methods of analysis.

2. American Public Health Association. 1960. Standard methods for the examination of dairy products, 11th edition. American Public Health Association, Inc., New York, New York.

3. American Public Health Association. 1967. Standard methods for the examination of dairy products, 12th edition. American Public Health Association, Inc., New York, New York.

4. Babel, F. J., E. B. Collins, J. C. Olson, I. I. Peters, G. H. Watrous, and M. L. Speck. 1955. The standard plate count of milk as affected by the temperature of incubation. J. Dairy Sci. 38:499-503.

5. Huhtanen, C. N., A. R. Brazis, W. L. Arledge, E. W. Cook, C. B. Donnelly, R. E. Ginn, J. N. Murphy, H. E. Randolph, E. L. Sing, and D. I. Thompson. 1970. Effect of dilution bottle mixing methods on plate counts of raw milk bacteria. J. Milk Food Technol. 33:269-273.

6. Huhtanen, C. N., A. R. Brazis, W. L. Arledge, E. W. Cook, C. B. Donnelly, S. E. Ferrell, R. E. Ginn, L. Lembke, D. J. Pusch, E. Bredvold, H. E. Randolph, E. L. Sing, and D. I. Thompson. 1970. A comparison of horizontal versus vertical mixing procedures and plastic versus glass petri dishes for enumerating bacteria in raw milk. J. Milk Food Technol. 33:400-404.

7. Johns, C. K. 1959. Applications and limitations of quality tests for milk and milk products. A review. J. Dairy Sci. 42:1625-1650.

8. Nelson, F. E., and M. P. Baker. 1954. The influence of time and temperature of plate incubation upon bacterial counts of market milk and related products, particularly after holding under refrigeration. J. Milk Food Technol. 17:95-100.

9. Pedraja, R., and A. Mengelis. 1964. Effect of time and temperature of incubation on the plate count of dry milks. J. Milk Food Technol. 27:241-244.